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***Efficiency and safeness improvement and
cost containment strategy in Assisted Reproduction
Technique (ART)***

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INTRODUCTION

38 years ago it was announced to the world the birth of Louise Brown, first child conceived in a test tube, by British scientist Robert Edwards ' Nobel Prize in medicine in 2010.

Today infertility affects approximately 15-20% of couples in the world, which globally means 50-80 million people. Latest worldwide estimates indicate that between 2008 and 2010, 4.461.309 cycles were initiated (Dyer et al., 2016), resulting in the estimated birth of 1.144.858 babies, up to 5% of all births in some countries (Kupka et al., 2016). The estimated overall number of initiated cycles and of babies born increased by almost 9.5 and 9.1% per annum, respectively, during the 3-year period.

The ongoing global expansion of ART can be attributed both to increased utilization within countries where ART is well established as well as the adoption of the technology into previously ART naïve countries.

According to the Italian Assisted Reproduction Technology Register (IARTR), the trends of ART initiated cycles per million inhabitants and per million women of reproductive age (between 15 and 45 years) were constantly growing from 2005 to 2014, with an increase of 466 cycles (+ 73.3%) and of 3,172 cycles (+ 118.2%), respectively. (IARTR registry, 2016). Over the years, progress has been made in the field of Reproductive Medicine, passing from the first treatments made of spontaneous cycle, to the introduction of medical strategies of multiple follicular growth stimulation, which is associated to an improvement of outcomes in terms of the number of mature oocytes retrieved, pregnancy rate and live birth rate.

The next step was the introduction in clinical practice of the hormone GnRH analogues, drugs with the aim of preventing premature LH peak, due to cancellation of several cycles. To this is associated the need to control the IVF complications, such as the hyperstimulation ovarian syndrome (OHSS).

The clinical introduction of GnRH antagonists, at the end of the years ' 90, opened new perspectives in ovarian stimulation strategy. Clinical data obtained from randomized controlled trials have shown the benefits of using these drugs in terms of lower dose of gonadotropins, shorter duration of treatment, reduced rates of OHSS, with an overall reduction of costs (Al-Inany HG et al., 2011). Also permitted the use of GnRH agonists for induction of oocyte final maturation, in order to reduce as much as possible OHSS rates.

The ovarian response to stimulation with exogenous gonadotropins during IVF is a critical determinant of live birth rates and adverse outcomes (R.G. Steward et al., 2014; Sunkara et

al., 2011). Healthcare providers and national guidelines recognize the need for individualization of the starting dose of gonadotropin by using predictive factors related to patient characteristics and diagnostic markers of ovarian reserve to attain an optimal oocyte yield while minimizing the risk of an excessive response and OHSS. In practice, clinicians are required to individualize treatment according to their own experience, using subjective preferences for predictive parameters, because there is not a standard position regarding which factors to take into account or the weight of each factor when determining the dose. The considerable individual heterogeneity in ovarian response to the same dose of gonadotropin, the limited performance of baseline patient characteristics including age, FSH, and antral follicle count (AFC) in predicting ovarian response, and their inconsistent clinical interpretation, as well as the lack of validated dosing algorithms, have limited the generalizability of efficacious and safe ovarian stimulation (Fauser et al., 2008).

Ovarian hyperstimulation syndrome (OHSS) is an uncommon but serious complication associated with controlled ovarian stimulation during assisted reproductive technology (ART). The incidence of OHSS varies between different types of fertility treatment, with treatments involving greater degrees of ovarian stimulation being associated with a higher incidence. In cycles of conventional in vitro fertilisation (IVF), mild OHSS has been estimated to affect around one-third of cycles, while the combined incidence of moderate or severe OHSS varies from 3.1% to 8.

The 14th European IVF-Monitoring report, analysing data from 25 European countries, found an incidence of hospitalization due to OHSS of 0.3% in 2010. Data from the USA showed OHSS to be the commonest complication of IVF treatment with an incidence of moderate or severe OHSS in 2011 of 1.1%.

OHSS is rare following ovulation induction with clomifene, or monofollicular ovulation induction with gonadotropins, but it has been reported. Very rarely, OHSS may occur spontaneously, in association with pregnancy.

This research work was carried out in order to identify the best strategies to improve the effectiveness and safety of IVF treatments. The goals are:

1. To assess how the estradiol serum levels, in the late ovarian stimulation phase, are predictive of IVF outcomes, in terms of number of oocytes retrieved, good quality embryos, pregnancy rate and live birth rate
2. To assess GnRh agonist dose for the trigger of final oocyte maturation in order to reduce ovarian hyperstimulation (OHSS) rate

3. To evaluate the possible long term IVF risks (metabolic, cardiovascular and oncological risks) in order to improve safeness
4. A cost reduction in IVF, by the reduction of cycle cancellation rate due to OHSS risks or E2 level drop, and hospitalization rate for ovarian hyperstimulation syndrome (OHSS).

The Research Project

The research project was carried out with the purpose to identify the best strategies to improve the efficiency and safety of Assisted Reproduction Treatments (ART), then the health care spending costs.

Target 1

Effectiveness optimization of controlled ovarian stimulation treatments aimed at IVF in terms of improvement in ovarian response to hormonal stimulation (oocyte retrieval, embryo quality, pregnancy rate)

- Development of a mathematical model predictive of ovarian response to stimulation
- Analysis of the dynamic changes of serum E2 in the late phase of COH in IVF
- Evaluation of the relationship between these changes and the intermediate and final outcomes of COH in IVF

Target 2 (realized at the Instituto Valenciano de Infertilidad (IVI) in Barcelona)

Improving the safety of COH treatments, in order to prevent IVF complications

- **Triggering with different doses of gonadotropin releasing hormone (GnRh) agonist in oocyte donor cycles: a randomized clinical trial (RCT)**
- **Long term COH complications: metabolic, cardiovascular and oncological risk evaluation. An observational, retrospective study**

Chapter 1: IN VITRO FERTILIZATION

1.1 The steps of in vitro fertilization

The first attempts to create artificial animal insemination date back to the seventeenth century, with failed attempts to M. Malpighi (1628-1694) to fertilize the silkworm, and continue with the experiments of Jacopi Wiltheim on salmon and trout eggs and those of the Swedish Clerk on the spider.

In 1782, the Italian Lazzaro Spallanzani made the first artificial insemination in dogs; 3 puppies are born after 62 days. Thouret in 1785 announced that it had artificially inseminated his wife successfully. In 1866 the gynecologist M. Sims reports a pregnancy of 55 attempts at intrauterine insemination. Finally, in 1884 Pancoast announced to have successfully performed the first artificial insemination with donor sperm.

From the end of the nineteenth century appear the first experiments to obtain the formation of an embryo directly in the laboratory. In 1890 Professor W. Heape reported successful embryo transfer from a donor to a recipient female rabbit, with the establishment of a full-term pregnancy, representing the first example of a surrogate pregnancy. In 1944, two American biologists, Rock and Menkin, describe successful in vitro fertilization of human eggs and their development stage of embryos for two three cells. In 1969, Edwards, in England, he obtained human embryos by performing in vitro fertilization. In 1976 Steptoe and Edwards got the first pregnancy from IVF, unfortunately ectopic; on November 10, 1977, they performed the transfer of an eight-cell embryo and on July 25, 1978, at 23:57, at a hospital in Oldham (Manchester), born Louise Joy Brown, the first test-tube baby conceived after more than 600 attempts, . On January 11, 1982 in a private clinic in Naples, born Alessandra Abbisogno the first Italian child conceived through IVF. The On December 20, 2006, in Bristol, Louise becomes a mother, giving birth to a child (conceived *naturally*). In 2010, Edwards was awarded the Nobel Prize in Physiology or Medicine "for the development of in vitro fertilization".

In just over 30 years, in the world, more than 3 million children conceived through IVF. were born. In Italy, according to data of the Ministry of Health, in 2008 approximately 59,000 couples have undergone assisted reproduction techniques for a total of 80,000 initiated cycles, 12,767 pregnancies and 10,825 children born.

1.2 The Development of Ovarian Stimulation Agents

Evidence of the endocrine pituitary-gonadal axis arose early in the 20th century when it was observed that lesions of the anterior pituitary resulted in atrophy of the genitals. The first convincing evidence supporting the existence of two separate gonadotropins (initially referred to as Prolan A and Prolan B) was provided by Fevold *et al.* in 1931 (1), and both LH and FSH were subsequently isolated and purified. In 1928, Ascheim and Zondek (2) described the capacity of urine from pregnant women to stimulate gonadal function. The concept of stimulating ovarian function by the exogenous administration of gonadotropin preparations has intrigued investigators for many decades. In 1940, Hamblen (3) reported the ability of purified pregnant mare serum to induce ovulation in humans by iv administration. However, these early attempts had to be stopped due to species differences and resulting antibody formation impacting on efficacy and safety. Clinical experiments in the late 1950s demonstrated that extracts derived from the human pituitary could be used to stimulate gonadal function (4). Subsequently, experiments involving the extraction of both the gonadotropic hormones LH and FSH from urine of postmenopausal women led to the development of human menopausal gonadotropin (hMG) preparations. From the early 1960s, these were used for the stimulation of gonadal function in the human (5). A second important development allowing for ovarian stimulation on a large scale arose when the first estrogen antagonist tested in cancer patients was found to induce ovulation.

1.2.1 The discovery of clomiphene citrate

In the late 1950s, the first nonsteroidal estrogen antagonist (MER-25) was tested for the treatment of breast cancer and endometrial hyperplasia. The administration of CC in women with endometrial hyperplasia suffering from secondary amenorrhea was followed by the recommencement of menstrual cycles (6). Shortly thereafter, the ovulation-inducing capacity of a closely related antiestrogen (MRL/41) was recognized (7).

CC was originally developed for clinical use by the Merrel company in 1956. Nearly 50 yr later, it is still considered to represent the first line treatment strategy in most anovulatory infertility and is still the most applied drug for infertility therapies worldwide.

CC is an oral antiestrogen consisting of a racemic mixture of two stereoisomers. Stimulation of ovarian function is elicited by raised pituitary FSH secretion due to blockage of E₂ steroid feedback by CC. Overall, a 50 – 60% increase of serum FSH levels above baseline has been described (8-10). The exact nature of the mechanism of action of CC is still uncertain (8-10), but induced changes in the IGF system may also be important (10). CC for ovulation induction in anovulatory women is considered to be relatively safe because steroid negative feedback remains intact. The oral route of administration and low costs represent additional advantages of this preparation. After the first IVF baby born in a natural cycle (11), four normal IVF pregnancies were subsequently reported after ovarian stimulation with CC (12). In the following years, many groups reported IVF results after CC, with or without gonadotropin cotreatment (13,14).

1.2.2. Gonadotropins

Human menopausal gonadotropins first became widely used for IVF in the United States (15,16). For over two decades, gonadotropin preparations have also been extensively applied for ovarian stimulation in ovulatory women for empirical treatment of unexplained subfertility. The aim is to increase the number of oocytes available for fertilization *in vivo* (17).

The initial preparations were very impure with many contaminating proteins; less than 5% of the proteins present were bioactive. However, improved protein purification technology allowed for the production of hMG with reduced amounts of contaminating nonactive proteins and eventually the development of purified urinary FSH (uFSH) preparations by using monoclonal antibodies since the early 1980s (5). The currently available pure products allow for less hypersensitive reactions and less painful sc administration. Due to the worldwide increased need for gonadotropin preparations, demands for postmenopausal urine increased tremendously, and adequate supplies could no longer be guaranteed.

Through recombinant DNA technology and the transfection of human genes encoding for the common α - and hormone-specific β -subunit of the glycoprotein hormone into Chinese hamster ovary cell lines (21), the large scale *in vitro* production of human recombinant FSH (recFSH) has been realized (22,23). The first pregnancies using this novel preparation in ovulation induction (24) and in IVF (25,26) were reported in 1992. Since then, numerous, large-scale, multicenter studies have been undertaken demonstrating their efficacy and safety. The recombinant products offer improved purity, consistency, and large-scale availability. Because of its purity, recFSH can now be administered by protein weight rather than bioactivity, and so-called “filled- by-mass” preparations are now available for clinical use. During recent years, recombinant LH (recLH) and hCG (rechCG) have also been introduced for clinical application.

1.2.3. GnRH agonists

In 1971, the small decapeptide GnRH was isolated, and its structure was elucidated (25,26). This decapeptide is secreted by the hypothalamus into the portal circulation in an intermittent fashion stimulating the pituitary gonadotropes to synthesize and secrete LH and FSH (27).

In 1978, it was discovered that repeated administration of GnRH agonists produced a transient increase in gonadal function followed by a decrease in gonadal function and a significant fall in sex steroids (28,29). Although initial binding to GnRH receptors results in activation, continuous occupation leads to desensitization due to the clustering and internalization of pituitary GnRH receptors, resulting in falling LH and FSH levels (30). If the agonists are administered for a period of several months, LH levels remain suppressed, but FSH levels return to normal and eventually rise to supraphysiological levels (31).

Pulsatile administration of GnRH was established as an effective and safe means of treating hypogonadotropic hypogonadal anovulation (29-32). The first reports concerning its use for the prevention of a premature LH rise during ovarian stimulation appeared in the early 1980s (33-35). During initial studies with hMG stimulation of multiple follicle development for IVF, it became apparent that a premature LH peak occurred in 20–25% of cycles due to positive feedback activity by high serum E₂ levels during the mid- follicular phase of the stimulation cycle (35).

This advanced exposure to high LH was associated with premature luteinization of follicles and either cycle cancellation due to follicle maturation arrest or severely compromised IVF outcomes. The clinical development of GnRH agonists allowed for the complete suppression of pituitary gonadotropin release during ovarian stimulation protocols for IVF (33, 36–38). Induced pituitary down-regulation indeed resulted in significantly reduced cancellation rates and improved overall IVF outcome (39). Moreover, the approach of GnRH agonist cotreatment facilitated scheduling of IVF and timing of oocyte retrieval.

In the long protocol, GnRH agonist treatment usually commences in the luteal phase in the preceding cycle and is continued until hCG administration. Due to the intrinsic agonist activity of the compound, pituitary down-regulation is preceded by an initial stimulatory phase (referred to as the “flare” effect). This flare effect renders the approach of GnRH agonist long protocol for ovarian stimulation time consuming, because ovarian stimulation can only commence when pituitary quiescence has occurred, usually around 2 wk after commencing treatment (40). It is uncertain whether ovarian response to exogenous stimulation is affected by GnRH agonist cotreatment (41), and some women suffer from serious hypoestrogenic side effects, such as mood changes, sweating, and flushes.

The “short” or “flare-up” protocol combines GnRH agonist therapy, started at cycle day 2, with gonadotropins initiated one day later (42). The immediate stimulatory action of the GnRH agonist serves as the initial stimulus for follicular recruitment. Adequate follicular maturation is on average reached in 12 d, which should allow enough time for sufficient pituitary desensitization to prevent any premature LH surges (43).

Several investigators have tried to shorten the duration of GnRH agonist administration by early cessation, because pituitary recovery after cessation takes around 14 days (44). The GnRH agonist is started in the midluteal phase of the preceding cycle and discontinued during or even before the FSH treatment is started. Several prospective randomized controlled studies have been performed comparing this approach with the long protocol (44–48). Although premature rises in LH did not occur (confirming delayed pituitary recovery from desensitization), no clear clinical benefit has been demonstrated by this approach.

A meta-analysis comparing short and long IVF protocols showed a higher number of oocytes retrieved and higher pregnancy rates in the long protocol, although more units of gonadotropin were needed (49). In terms of gonadotropin suppression and number of retrieved oocytes, the midluteal phase of the preceding cycle is the optimal moment for the initiation of the GnRH agonist, in comparison to the follicular, early, or late luteal phase (50,51). A major clinical advantage of the long protocol of GnRH agonist administration is the contribution to the planning of the oocyte retrieval because the initiation of exogenous gonadotropins after pituitary desensitization can be delayed, without a detrimental effect on IVF outcome (52, 53). A potential disadvantage with the luteal phase initiation of GnRH agonist is that spontaneous pregnancy present at the time of commencing treatment cannot be excluded with certainty. The extensive evidence supporting the long protocol has led to its widespread adoption as the standard of care (49). However, the recent clinical introduction of GnRH antagonists may ultimately lead to a new standard of care in IVF practice.

1.2.4. GnRH antagonists

GnRH antagonists may be administered at any time during the early or midfollicular phase of a treatment cycle to prevent a premature LH rise. Several studies have been performed to determine the minimal effective dose and treatment schedule in IVF patients (54, 55). Two general approaches have emerged.

In the single-dose protocol, one injection of 3 mg cetrorelix (ganirelix is not provided in this depot formulation) is administered in the late follicular phase on stimulation day 8 or 9. This is sufficient to prevent a LH surge in 80% of women (55).

In the multiple-dose GnRH antagonist protocol, 0.25 mg cetrorelix or ganirelix is given daily from the sixth day of gonadotropin stimulation onward (54, 56). The rationale behind starting GnRH antagonist at least 5 d after commencing stimulation with gonadotropins is based on the reduced possibility of observing a premature LH rise in the early follicular phase (57).

Four large, industry-sponsored, prospective multicenter clinical trials comparing daily GnRH antagonist injections with long GnRH agonist protocols in IVF patients undergoing ovarian stimulation have been reported (58-60). With a GnRH antagonist, the duration of gonadotropin treatment is shortened by 1–2 d, and slightly fewer follicles are seen at the time of hCG injection compared with a GnRH agonist. Therefore, the number of recovered oocytes tends to be lower. In these studies, no significant difference was found with respect to percentages of metaphase II oocytes, fertilization rates, and number of good quality embryos. Pregnancy rates were adequate in both groups in all four studies, but in every one the absolute rate was lower in the GnRH antagonist group. A meta-analysis of five large randomized trials showed an overall decrement in pregnancy rate of 5% (odds ratio, 0.75; 95% confidence interval, 0.62–0.97) (62)

It has been hypothesized that the lower observed pregnancy rates may be a consequence of the currently advised treatment regimen. It has been suggested that the larger numbers of oocytes and embryos with agonists allow better selection, although the numbers of good quality embryos do not seem to be different. The GnRH antagonist was started on a fixed day of stimulation (d 6) in these studies, which may be too early for some patients and may lead to a diminished number (and quality) of oocytes.

Studies comparing the *fixed antagonist protocol with a flexible protocol*, in which the daily antagonist administration is started when at least one follicle reached a size of 14 mm, showed no differences in IVF outcome, except that the dose of GnRH antagonist was reduced in the flexible protocol (63). When GnRH antagonist is commenced, there appears to be no requirement to increase the dose of FSH (64,65) or supplement LH (66). Commencing GnRH antagonist in the late follicular phase enables the endogenous FSH rise to be harnessed to commence ovarian stimulation and then supplemented by exogenous gonadotropin stimulation from the midfollicular phase onward to achieve multifollicular development (67). This approach is cost-effective and patient-friendly alternative to standard stimulation regimens.

Based on the inverse association between implantation rates and ganirelix dose in the higher dosage groups in the large dose-finding study (68), direct effects of GnRH antagonists on human embryos have been suggested. Adverse effects were not observed on the freeze-thaw embryos of these cycles (69). Moreover, retrospective comparison of pregnancy rates after transfer of frozen-thawed two-pro-nucleate oocytes obtained in either a long GnRH agonist

protocol (or a GnRH antagonist protocol) showed no differences in implantation, pregnancy, or miscarriage rates (70).

The availability of gonadotrophin-releasing hormone (GnRH) antagonists for ovarian stimulation protocols has generated many meta-analyses comparing it to GnRH agonist long protocols. (71-75). These meta-analyses have yielded conflicting results for pregnancy rate, with a tendency toward a better outcome for GnRH agonists. Recently, a Cochrane review seems to have settled the conflicts by demonstrating no evidence of statistically significant differences in the rates of live births or ongoing pregnancies when comparing GnRH agonist long protocols with GnRH antagonist protocols. Furthermore, the use of the GnRH antagonist protocol has made possible the use of GnRH agonist for the final trigger, minimizing the risk of hyperstimulation ovarian Syndrome (OHSS).

Diagrammatic representation of different IVF protocols

Days -7 -6 ≈ -1 0 1 2 3 ≈ 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22

GnRHa FSH only hCG Oocytes collection
Ultrashort

GnRHa GnRHa + FSH hCG Oocytes collection
Short

GnRHa GnRHa + FSH hCG Oocytes collection
Long, follicular protocol

GnRHa GnRHa + FSH hCG Oocytes collection
Long, mid-luteal protocol

FSH FSH + GnRH antagonist hCG Oocytes collection
Antagonist protocol

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CHAPTER 2 - TARGET ONE

Effectiveness optimization of controlled ovarian stimulation (COH) in IVF treatments terms of improvement in ovarian response to hormonal stimulation (oocyte retrieval, embryo quality, pregnancy rate)

- Development of a mathematical model predictive of ovarian response to stimulation
- Analysis of E2 serum dynamic changes in the late phase of the COH cycle
- Evaluation of the relationship between these changes and the intermediate and final outcomes of COH cycle

2.1 MATERIALS AND METHODS

We carried out a retrospective study of 1116 consecutive IVF homologous cycles at the Assisted Reproductive Unit of IRCCS Burlo Garofolo, Department of Medical, Surgical and Health Sciences, University Of Trieste (Italy) from January 2013 to January 2016. The data collected for each patient were: age at the time of the procedure; body mass index (BMI); third day hormonal dosage; number of the years of infertility; type of infertility (primary or secondary); cause of infertility (male factor, ovulatory factor, tubal factor, endometriosis, multiple factors, idiopathic infertility).

All the patients underwent ovarian stimulation (COH) with tailored protocol. For each cycle, the following variables were considered: data relating to the type of COH protocol used (GnRH agonist long protocol or GnRH antagonist flexible-dose protocol); the maximum estradiol (E2) serum concentration on the trigger day with hCG; the duration of stimulation (days) and gonadotropin total dose used.

A written consent form for all the patients who underwent an IVF treatment was required. All the patients consented to the use of anonymous data for research purposes.

Ovarian stimulation protocol

Starting on cycle day 2, the patient received 150 - 225 UI/die s.c. of recombinant- Follitropin (r-FSH) with or without human Menopausal Gonadotropin (hMG). Ovarian response was monitored with vaginal ultrasound and plasma estradiol. The first control was performed on day 5 of the cycle. The FSH or hMG dose was then adjusted according to the ovarian response. Recombinant or Human chorionic gonadotropin was given to induce ovulation when at least two follicles ≥ 18 mm in diameter were present.

Oocyte retrieval was performed 35 hours after the ovulation induction. IVF or ICSI technique were chosen, depending on the cause of infertility. The fertilization was determined by the presence of two pronuclei (2PN) using an invertoscopia the first day after insemination. The embryos were classified into grades I to V, based on the number of blastomeres and uniformity and the percentage of fragmentation, according to the classification of Veeck system. The embryo transfer procedure took place 48-72 hours after the oocyte retrieval. The luteal phase was supplemented with a daily dose of 50 mg progesterone in oil or 600 mg of vaginal micronized progesterone. On the 15th day after ovum pick up, pregnancy test was performed.

Statistical Analysis

We evaluated the 17β -E2 levels at 96 and 48 hours before eggs retrieval. The dynamic changes of serum E2 were evaluated by calculating the increase or decrease rate of the E2 at 96 h and 48 h prior to oocyte retrieval.

Then, we identified a coefficient of E2 increase or decrease, using a mathematical formula. In this way we were able to identify four different coefficient categories: a negative coefficient, with a negative increase (category A); a positive coefficient with an increase between 0 and 50% (category B), a positive coefficient with an increase between 50 and 100% (category C); a positive coefficient with an increase more than 100% (category D).

For each coefficient category, the correlation with the IVF outcomes was analyzed.

The final outcomes were: the total number of retrieved oocytes, the number of mature oocytes (metaphase II), the ratio of mature oocytes and total oocytes, the fertilization rate, the total number of embryos, the number of good quality embryos (grades I-II), the ratio between embryos of good quality and total embryos, the implantation rate, the clinical pregnancy rate and the live birth rate.

One-way ANOVA, Kruskal Wallis test and Dunn's Multiple Comparison Test were used for the analysis of the continuous variables, whereas for the categorical variables a Chi-square test was chosen. Statistical analysis was performed using GraphPad Prism 5 software.

2.2 RESULTS

In this retrospective study, we analysed 1116 consecutive IVF homologous cycles. The mean age of patients was 37.25 years (SD 4.19), the average BMI was 22.67 (SD 3.51), the value of FSH on day 3 of the menstrual cycle was 7.26 (SD 2 , 66). The majority of cycles was performed on patients with primary infertility (876/1116 cycles). The most common cause of infertility was male factor (613 cases out of 1116 cycles), followed by idiopathic factor (84/1116 cycles), tubal factor (71/1116 cycles), endometriosis (26/1116 cycles), ovulatory factor (19/1116 cycles); in 303 cases the cause was mixed (male and female factor).

<i>Variables</i>	<i>Mean</i>
Age (years)	37,25 y
BMI (kg/m²)	22,67
Basal FSH	7,26
Type of infertility	
Primary	876
Secondary	240
Causes of infertility	
Male factor	613
Ovulatory factor	19
Tubal factor	71
Endometriosis	26
Idiopathic	84
Mixed female and male factor	303
Days of COH	11,9
COH protocol	
Long GnRh agonist protocol	671
GnRh antagonist protocol	445
FIV-ET cycles	305
ICSI cycles	756

Of all performed cycles, in 671 we used a long agonist protocol and in 445 a GnRH antagonist flexible-dose protocol for the COH. Conventional FIV was carried out in 305 patients, while ICSI was carried out in 756 cases. The mean dose of gonadotropins used was 2707 IU (1464 IU SD); the mean duration of stimulation was 11.94 days (SD 3.53). The mean value of E2 at the trigger time was 1,490.57 (SD 797.86).

Oocyte retrieval was realized in 1085 cycles out of 1116 (for a total of 7426 oocytes retrieved): in 17 cycles no mature oocytes (MII) were retrieved.

In 13 cases out of 1116 we didn't perform the insemination, due to altered oocyte morphology (8/1116) or due to the absence of semen sample (5/1116). The fertilization rate was 67.68%.

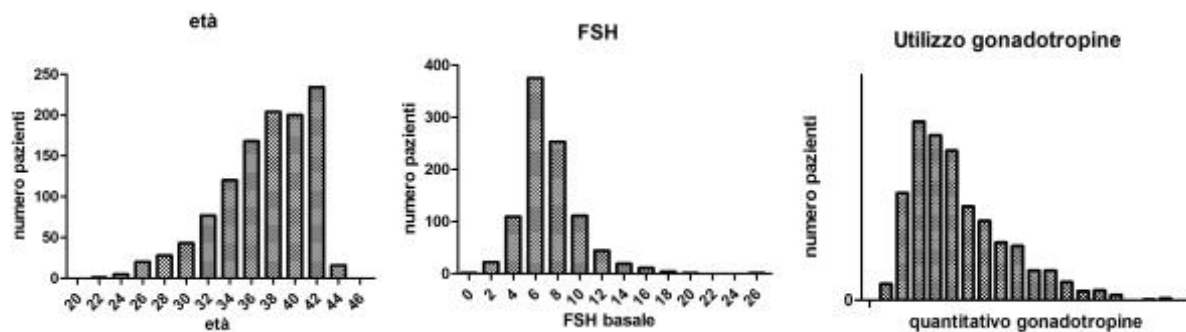
We carried out the embryo transfer (ET) only in 904 cycles out of 1116: in 197 an embryo degeneration before the ET was observed. A maximum of 3 embryos was transferred for a single cycle.

In 15 cases, we cryopreserved embryos to prevent the onset of the hyperstimulation ovarian syndrome (OHSS). The implantation rate was 11.26%; the pregnancy rate was 16.22% per OPU and 20,0% for ET; the live birth rate per OPU was 14.78%, while it was 18.25% per ET (table 1.)

TOTAL CYCLES	1116
No oocyte (n. cycles)	31
No M2 oocyte (n. cycles)	17
No insemination (n cicli)	61
No good quality oos (n cycles)	56
No sperm (n cycles)	5
Fertilization rate (%)	67,68
Embryo transfers (n cycles)	904
No ET for OHSS risk (n cycles)	15
Implantation rate (%)	11,26
Clinical pregnancy rate (%)	
Per OPU	16,22
Per ET	20,02
Live birth rate	
Per OPU	14,78
Per ET	18,25

Table 1

For all the cases we observed an asymmetric distribution for demographic characteristics (eg. age and the basal values of FSH) and for other parameters associated with COH, such as the doses of gonadotropins and the number of days of stimulation.



We identified a coefficient of E2 increase or decrease, using a mathematical formula.

$$E2 \text{ coefficient } (\%) = \frac{(E2 \text{ 48h before OPU} - E2 \text{ 96h before OPU})}{E2 \text{ 96h before OPU}}$$

Then, according to the E2 dynamics changes during COH, we observed 4 different categories of E2 increment coefficients of E2 values, with this patients distribution:

1. category A (decreasing trend in estrogen levels): 104 cycles out of 1116
2. category B (E2 increasing trend between 0 to 50%): 277 cycles out of 1116
3. category C (E2 increasing trend between 50 and 100%): 445 cycles out of 1116
4. category D (E increasing trend > 100%): 290 cycles out of 1116
- 5.

The patients distribution in each category is similar for BMI, but not for age and FSH basal values: in fact, there are significant differences between A and B and A and C groups if the age of the patients is compared. Significant differences are observed between the groups A and B and the B and D groups for the FSH basal values (table 2).

	Age		BMI		Day-3 FSH	
E2 coefficient	Mean	SD	Mean	SD	Mean	SD
A	35,86	4,77	22,45	3,49	6,66	2,15
B	37,34	4,17	22,46	3,36	7,70	2,84
C	37,56	4,14	22,78	3,50	7,23	2,67
D	37,22	3,97	22,65	3,67	7,12	2,95
<i>P value</i>	<i>0,0065</i>		0,4547		<i>0,0072</i>	

Table 2

Furthermore, we observed a significant association between a shorter COH duration and the E2 increase trend in 48 h greater than 100% (*group D; p <0.0001*) (Table 3).

STIMULATION DAYS				
E2 COEFFICIENTS	A	B	C	D
N cases	104	277	445	290
Median	12,00	12,00	12,00	12,00
25°-75° centile	11,00-13,00	11,00-13,00	11,00-13,00	10,00-12,00
Mean	11,98	12,60	11,94	11,30
SD	1,86	6,35	1,76	1,70

Table 3

We observed significant differences between the groups with different E2 coefficient of Increase ($p < 0.0001$). However, no differences were observed within the same category of E2 increase, if we compare long GnRh agonist protocol with GnRH antagonist one ($p > 0.05$). According to the COH protocol used, we observed statistically significant differences on the mean doses of the gonadotropins used ($p < 0.0001$). In particular, we found a lower use of gonadotropins in patients of category D (E2 negative increase).

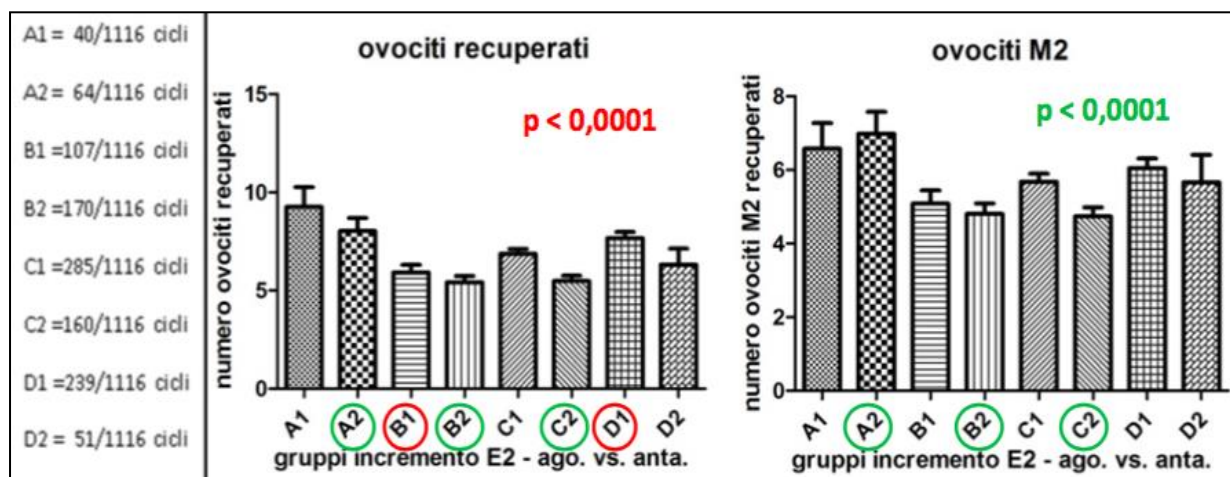
Regarding the total number of oocytes retrieved, we observed statistically significant differences between categories A and B, A and C, B and C, B and D ($p < 0.0001$); there are no differences in the total number of eggs retrieved between groups A and D, C and D ($p > 0.05$). Statistical differences were observed about the number of mature oocytes (MII) between categories A and B, A and C, B and D ($p < 0.0002$).

Regarding the total number of oocytes retrieved according to the protocol used, for the agonist protocol we observed a statistical difference between B and D group, in favour of D (E2 trend $> 100\%$); in the antagonist group, we observed a statistical correlation between A and B, and between A and C ($p < 0.0001$). We observed the same statistical correlation, for the antagonist protocol, when we analysed data about the mature oocyte retrieved (A and B, A and C).

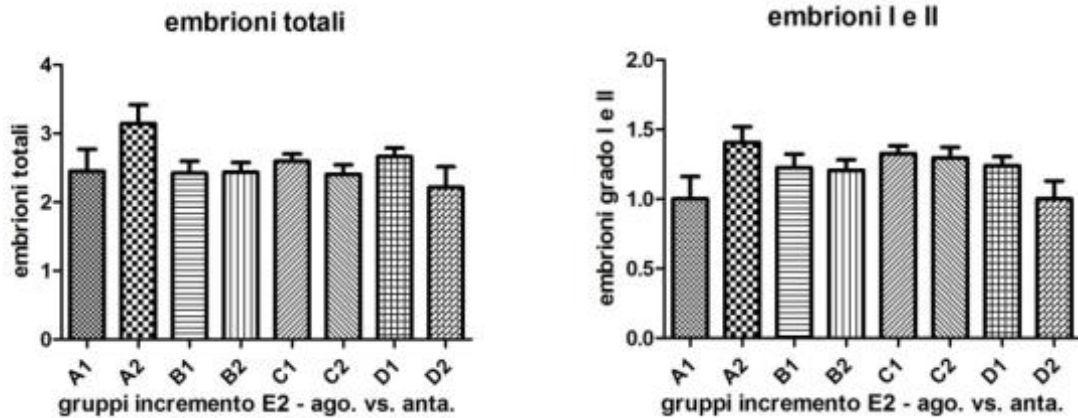
		A1	A2	B1	B2	C1	C2	D1	D2
	numero di cicli	40	64	107	170	285	160	239	51
ovociti totali	Mediana	9,5	7	5	4	6	5	7	5
	25% - 75% Percentile	4,00 - 12,75	4,00 - 11,75	3,00 - 7,00	2,00 - 7,00	4,00 - 9,00	3,00 - 7,75	4,00 - 11,00	2,00 - 8,00
	n. massimo ovociti	27	22	19	22	22	16	23	24
	Media	9,25	8,031	5,916	5,424	6,863	5,481	7,665	6,314
	SD	6,428	5,279	4,098	4,116	4,236	3,433	4,846	5,857
ovociti M2	Mediana	7	6	4	4	5	4	5	4
	25% - 75% Percentile	3,00 - 9,75	3,00 - 10,00	2,00 - 7,00	2,00 - 7,00	3,00 - 8,00	2,00 - 6,00	3,00 - 9,00	2,00 - 8,00
	n. massimo ovociti	19	20	18	21	19	14	18	20
	Media	6,575	6,984	5,084	4,806	5,674	4,738	6,05	5,667
	SD	4,414	4,712	3,699	3,709	3,745	3,083	3,991	5,328

T

Total oocyte and M2 oocyte according to COH protocol (agonist protocol 1; antagonist protocol 2)



We didn't observed any difference between the number of total embryos and different E2 growth trends, nor between good quality embryos (grade I and II) and different E2 growth trends.



Later, we compared the 4 different groups of E2 increase with the fertilization rate (FR), the implantation rate (IR), the clinical pregnancy rate (CPR) and live birth rate (LBR).

We found a significant difference in clinical pregnancy rate per embryo transfer (ET) between A and D categories ($p=0.0445$), but not for the other groups ($p>0.05$). When we compared the same categories A and D in terms of clinical pregnancy rate for ovarian pick up (OPU), live birth rate per OPU and live birth rate for ET we found a *P value* near to statistical significance (respectively 0.0727; 0.0938; 0.0593).

After a further analysis of the IR data, on the basis of the different E2 increase coefficients groups and the type of COH protocol, in the group A (decreased E2 levels) a difference in the IR between agonist (7 gs out of 46 embryos) and antagonist protocol (16 gs out of 102 transferred embryos) is observed ($p = 0.006$); the same difference was observed in the D group (E2 levels increased by 100%) (52 gs out of 383 embryos in the agonist protocol versus 3 gs out of 68 embryos transferred in the antagonists protocol) ($p = 0.042$).

In the GnRH agonist group we observed that the IR of group A is higher than the one observed in groups B, C and D (p value 0.004 respectively; 0.0002; 0.0002). In the GnRH antagonist protocol, we found a lower IR in group D than in group A, B and C (p value respectively 0.025; 0.022; 0.034).

We observed no differences in CPR per OPU and ET, and in LBR per OPU and ET.

2.3 DISCUSSION

Clinicians' ability to predict clinical outcomes before or during the course of IVF treatment is limited although numerous predictors are available, such as patients' age (1), basal FSH/LH ratio (2), embryo quality parameters (3,4) serum progesterone level (5,6) and estradiol (E2) level (7,8). Among these predictors, the serum E2 level is one of the most important factors because E2, a major product of granulosa cells, reflects the maturity of follicle and quality of oocytes (9,10). Accordingly, monitoring serum E2 level closely is the main way of evaluating the ovarian response, and serum E2 levels at different time points, such as basal E2 level, E2 level at the stimulation day 5 and on the day of hCG administration, as well as in the mid-luteal phase, have been documented in many papers as predictors of clinical outcomes (7,8,11–13).

Several authors have examined the effect of estradiol trends on IVF success, including the absolute value of day 5 E2 (3) Day 6 E2 (4), the trend of E2 from day 5 to the day of hCG, and the early response of E2 between day 0 and 6 (5).

Styer et al., in 2005 evaluated the effect of an unpredictable drop in serum estradiol prior to hCG administration on pregnancy outcomes in vitro fertilization cycles. The authors concluded that, in the absence of coasting, a drop in serum estradiol levels during GnRH-agonist downregulated controlled ovarian stimulation (COS) for IVF prior to hCG is not associated with a decrease in live birth rates or pregnancy loss rates.

Kim et al., in their study in 2010 investigated the effects of total E2 production during COS, calculated by “modified area under the curve for E2 (mAUC-E2)” and of the changes in E2 level during the initial stimulation period calculated as “slope of initial increase in E2 (SI-E2)” on the outcomes of IVF cycles using GnRH antagonist protocols. They concluded that the rate of initial increase in E2 rather than total E2 production during COS might affect the competence of retrieved oocytes in GnRH antagonist cycles. Surveillance on the initial change in E2 and subsequent modulation of stimulation could be beneficial in achieving optimal IVF outcomes in GnRH antagonist cycles.

Other studies suggest that E2 levels double in the late stages of IVF stimulation, meaning that there is a 100% increase every 2 days (14).

Our study aimed to identify serum E2 production variation during IVF controlled ovarian stimulation, through the development of a mathematic model able to identify the increasing and decreasing E2, in the late phase of ovarian stimulation, between 96 and 48 hours before ovarian retrieval.

The hypothesis was that E2 values follow a linear increase; to prove that we calculate the angular coefficient of the straight line, normalising this coefficient to the E2 value 96 hours before the oocytes retrieval: so, we used a dynamic index of E2 production that allowed us to analyse its role independently from the pure E2 levels.

$$\text{E2 coefficient (\%)} = \frac{(\text{E2 48h before OPU} - \text{E2 96h before OPU})}{\text{E2 96h before OPU}}$$

To evaluate the effects of a decrease or increase of E2 levels, we divided the population in 4 categories:

- Category A (E2 decrease)
- Category B (E2 increase between 0 and 50%)
- Category C (E2 increased between 50 and 100%)
- Category D (E2 increase >100%)

The index we used is different from the one used by other authors to evaluate the growth of E2 values: Shapiro et al. evaluated the difference between oestrogens levels the day of GnRH antagonist administration and the day of the second GnRH antagonist dose administration (17); Scotchie et al. examined the ratio of E2 serum levels before and after the antagonist administration (E2 after GnRH antagonist/E2 before GnRH antagonist), analysing the results in relation to the number of days of GnRH antagonist administration (16) Ranieri (18) and Ravhon (19) instead, considered estrogenic values after buserelin acetate administration, evaluating the difference between day 3 and day 2 of the menstrual cycle or, in other words, before and after the GnRH antagonist consumption. Kim et al. (15) considered the percentage of E2 level increase at the beginning of the cycle, considering the difference between the level at the first r-FSH administration and the 5th day of the stimulation, and moreover

they calculated the area under the curve of oestrogen levels at the beginning, middle and at the end of the stimulation.

There is a considerable difference between the studies in the criteria chosen to represent the dynamic changes of E2 levels, making difficult to compare the methods utilized and to evaluate their efficacy. Studies that evaluated late changes of oestrogen levels reported heterogeneous results; on the opposite, studies that analysed the effects of E2 changes in the first stages of stimulation agree on the possible correlation between dynamic index and outcome of the IVF procedure (15,19). Therefore, it is possible that changes of the E2 production in the first days of the stimulation correlate with oocytes quality at retrieval, meaning that monitoring E2 levels after COH is a poor predictor of the number of oocytes collected. However, serial monitoring in the advanced stages of stimulation allows better control of the final follicular recruitment, adjustments of drugs dose in case of hyper response to the therapy and to evaluate the effects of subsequent therapeutic changes.

Analysis of the data showed that a decrease in oestrogen levels is not frequent in the general population under IVF treatment: in our study it happens in 9% of the population study, a result similar to the 6% reported in the literature (17) Furthermore, we observed that lower E2 levels are usually present in younger patients, that they require lower mean doses of gonadotropin and had a good total number of retrieved and mature oocytes compared to the general population, but the implantation and pregnancy rate is the same of the patients that do not have a decreasing level of E2; we also saw that patients with a E2 increasing level more than 100% are characterized by, for the same age, lower need for gonadotropin assumption and higher retrieval of mature oocytes compared to patient with a E2 increment of 100% or lower, but the implantation and pregnancy rate is lower if GnRH antagonist was used.

Our data differ from what observed by Scotchie et al. (16) as these authors found a higher oocytes retrieval after administration of GnRH antagonist: in their study, patients with lower or steady trend of E2 levels were significantly older, required a higher mean use of gonadotropin compared to the general population, but E2 levels were lower in the 48 hours before the oocytes collection. In our study, the small number of patient in the group with a lowering trend might have influenced the results. However, it is possible that younger patients, characterized by a bigger ovarian reserve, may request lower gonadotropin doses throughout the stimulation but at the same time they could need more frequently changes in gonadotropin dose based on the follicular response, resulting in drastic changes of E2 levels at the later stages of stimulation, with a decreasing or rapidly increasing trend. Since the younger age, the possibilities of an on-going pregnancy are higher.

Moreover, it is possible that the higher increase of E2 levels at the time of ovulation induction might affect also the endometrial quality with consequences on the implantation and pregnancy rate.

We also analysed the relationship between dynamic changes of serum estradiol and IVF results in relation to the stimulation protocol chosen (GnRH agonist vs GnRH antagonist). Regarding GnRH agonist, we found that gonadotropin administration was lower in the groups with extreme changes in E2 levels. We observed differences in oocytes retrieval between group B and D but not in relation to other groups. Differences in E2 increase didn't affect results in term of mature oocytes retrieved, number of total embryos at the time of embryo transfer and number of good quality embryos.

If we compare different increasing trend of E2 based on the GnRH protocol used, we found that the implantation rate is higher in the lowering E2 levels group; if we compare the implantation rate in the agonist and antagonist group we see that it is higher in group A and D. These data indicates that in the GnRH protocol, the increasing E2 trend at the end of ovarian stimulation does not impact on the outcome of the cycle.

Our data are similar to the one of Simon (20) who found that there is no relationship between E2 levels and embryonic quality. In our study we didn't observe any relationship between E2 levels and pregnancy rate: it is possible that, to predict the outcome of ovarian stimulation with GnRH agonist, the absolute E2 level at the time of hCG administration is far more important than the oestrogens trend; this conclusions is supported also by some studies that find an impact of the E2 levels on the endometrial receptivity (20,21)

In our population we found that, if GnRH antagonists were used, the number of the total oocytes and mature oocytes collected was higher in the group with a negative coefficient of E2 levels, but there were no differences in regard of total number of embryos and good quality embryos. As already said, it is possible that this is the consequence of a younger age population and thus more responsive to ovarian stimulation, both in qualitative and quantitative terms. Furthermore, this result support our thesis that a lowering trend of E2 levels in the final days of the stimulation is not always a critical reason to withdraw the cycle, contrasting with what reported previously in the literature. (16).

No differences in reduction of implantation and pregnancy rate were found in the lowering E2 level group; we also saw that the implantation rate was lower in the group of patient with an increase of E2 levels higher than 100% of the starting level, most likely because of a detrimental effect on the endometrium.

Considering that GnRH administration gives an instant suppression of endogen LH, it was speculated that it could affect also E2 levels. Although data are limited, some clinicians suggested that an abnormal E2 pattern (plateau, decrease) after antagonist administration might affect the clinical outcomes (23). In our data, not only we didn't find any difference in the clinical outcomes based on the different oestrogen increasing levels, but we also found that a decreasing E2 levels was not frequent. Moreover, the absence of relationship between implantation rate, pregnancy rate and live birth rate may be a consequence of the different effect of GnRH antagonist on the endometrial quality.

Our data show that, in case of ovarian stimulation with GnRH antagonist, the E2 patterns with a decreasing or plateau trend do not affect the clinical outcomes of the IVF procedures, as in Shapiro and Scotchie studies (16,17). But our data are in contrast with Lindheim conclusions (24) who claims that a drop in E2 levels, after GnRH antagonist administration, affects the IVF outcomes negatively, also in case of an egg donor cycle.

With this study we were able to highlight that dynamic changes of estradiol levels during ovarian stimulation don't affect the outcome of IVF procedure.

Our results are in line with other few studies that analysed the relation between the index used during the controlled ovarian stimulation and the IVF procedure results. The majority of the reported data were obtained from the analysis of small population groups, which not always represent the entire sample of women seen in II and III level IVF centres.

This study, though the limited nature of a retrospective study, is characterized by a good sample of population analysed and by the study of dynamic changes of oestrogen levels subsequent to the ovarian stimulation with GnRH agonist (long protocol) or with GnRH antagonist.

Our analysis emphasizes the importance of the real usefulness of serum estrogen levels monitoring during ovarian stimulation.

Previously, it was highly recommended to constantly monitor the patient response to drug stimulation, suggesting that the dynamic modifications of estrogen levels were reflecting the ovarian response to the stimulation. But, with our study, we found that there is no relation between dynamic changes and IVF outcome and that stopping the stimulation before the oocytes retrieval, in case of lowering level of final E2 levels, should not be recommended. Moreover, since there are no significant differences between IVF outcomes in patient with different trends of increasing E2 levels, the routine of serial E2 levels monitor extended to all patients, regardless their characteristics, doesn't add any benefits and can increase the cost of the procedure.

In order to reduce health financial costs we could rather monitor the follicular growth with serial scans and serial E2 levels assessment limited to patients at high risk of OHSS, to strictly follow the response of these patients and to closely vary the therapy; on the contrary, serial scans could be the only method of monitoring in patients with normal or poor ovarian response.

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CHAPTER 3 – TARGET 2

Improving the safety of COH treatments aimed at IVF, in order to prevent IVF complications

3.1 Triggering with different doses of gonadotropin releasing hormone (gnrh) agonist in oocyte donor cycles: a randomized clinical trial (RCT).

3.1.1 GNRH AGONIST TRIGGERING: A REVIEW OF LITERATURE

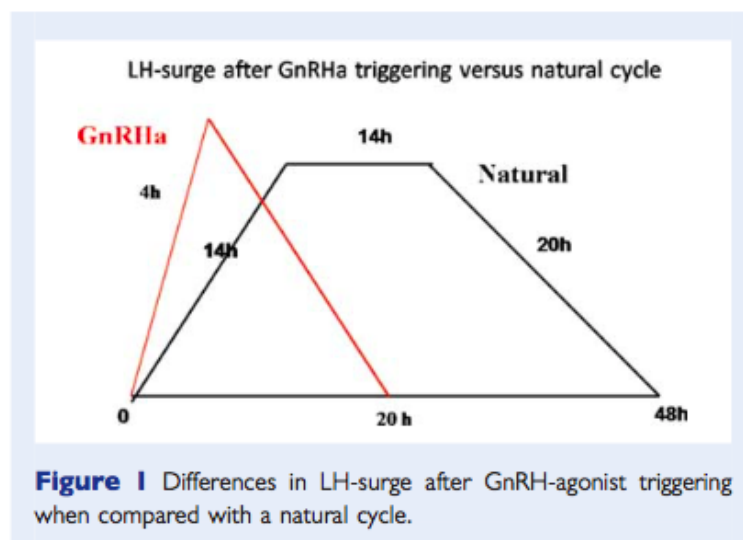
The administration of HCG to induce final oocyte maturation has been used for decades and has been considered the gold standard during ovarian stimulation for IVF cycles. Recently, however, it has been suggested that the time has come for a paradigm shift in triggering policies (Humaidan and Alsbjerg, 2014; Humaidan and Polyzos, 2014). Although HCG effectively induces oocyte maturation and maintains excellent pregnancy rates during the IVF process, the prolonged half-life of HCG compared with natural LH promotes supra-physiological luteal steroid levels and the development of multiple corpora lutea, resulting in a potential increased risk of ovarian hyperstimulation syndrome (OHSS). Therefore, the use of alternate modalities to induce oocyte maturation to prevent OHSS, such as gonadotropin releasing hormone agonist (GnRHa) has been the focus of research for years.

In 1988, Itskovitz et al. for the first time published a case report in which they describe the use of GnRH-agonist in two patients that in the previous cycle had developed ovarian hyperstimulation syndrome (OHSS) after hCG treatment; none of the them in developed signs of OHSS in this cycle.

The GnRHa trigger concept gained some interest in the late 1980s and early 1990s. However, with the introduction of GnRHa for pituitary down-regulation prior to IVF/ICSI treatment (Porter et al., 1984), this concept was clearly not applicable, as the simultaneous use of GnRHa for down-regulation and triggering of final oocyte maturation is not possible.

With the introduction of the GnRH antagonist (Albano et al., 1997; Borm and Mannaerts, 2000; Itskovitz-Eldor et al., 1998), it became again possible to trigger ovulation with a bolus of GnRHa as an alternative to HCG, as GnRHa will displace the GnRH antagonist from the GnRH receptor in the pituitary and elicit a surge of gonadotropins (LH and FSH). However, there are significant differences between the GnRHa-induced surge of gonadotropins and that

of the natural cycle. Thus, the LH surge of the natural cycle is characterized by three phases, with a total duration of 48 h (Hoff et al., 1983), as compared with the GnRHa-induced LH surge, which consists of two phases for a total of 24–36 h (Itskovitz et al., 1991).



This leads to a significantly reduced total amount of gonadotropins released from the pituitary when GnRHa is used to trigger final oocyte maturation (Gonen et al., 1990; Itskovitz et al., 1991). This per se could induce a defective luteal phase (Balasch et al., 1995; Segal and Casper, 1992), which needs a modification of the standard support in order to prevent a negative IVF outcome (Humaidan et al., 2010).

However, a possible advantage of GnRHa for triggering of final oocyte maturation in comparison with hCG is the simultaneous induction of a FSH surge comparable to the surge of the natural cycle. The role of the mid-cycle FSH surge in the natural cycle is not fully understood, but FSH has been shown to induce LH receptor formation in the luteinizing granulosa cells thus optimizing the function of the corpus luteum. Moreover, FSH specifically seems to promote oocyte nuclear maturation, i.e. resumption of meiosis (Zelinski-Wooten et al., 1995; Yding Andersen et al., 1999) and cumulus expansion (Stickland and Beers, 1976; Eppig, 1979).

Interestingly, several studies reported the retrieval of more mature oocytes after GnRHa trigger, which could be an effect of a more physiological surge including a FSH surge as well as an LH surge (Imoedemhe et al., 1991a; Humaidan et al., 2005, 2009a, 2010; Oktay et al., 2010).

Several studies, reported a reduction of OHSS incidence in the egg donation program: it has been demonstrated through retrospective studies (BS Shapiro et al., 2007; Hernandez ER et al., 2009) and randomized clinical studies (Acevedo B. et al. , 2006; Galindo et al., 2009).

However, until now the greatest advantage of GnRHa triggering in ovarian hyperstimulation, is the total elimination of OHSS. This has led to the fact that GnRHa triggering is now the method of choice in many oocyte donation programs, resulting in a high good quality oocyte yield, an elimination of OHSS, a higher degree of patient convenience and an excellent pregnancy rate in recipients (Hernandez et al., 2009; Bodri et al., 2009).

Currently, we don't know what is the optimal dose of GnRH agonist.

In 1996, Parneix et al, compared in the same patients undergoing IVF, different doses of different molecules of GnRH-agonist, with different routes of administration (subcutaneous injection and nasal spray). Subsequent studies used single doses of a same GnRH-analogue, such as Triptorelin 0.2 mg, Buserelin 0.5 mg, Leuprolide acetate 1 mg and 1.5 mg (Bodri et al., 2009; Hernandez et al., 2009; Papanikolaou et al., 2011, Humaidan et al., 2010; Castillo et al., 2010).

The main purpose of our randomized clinical trial was to compare different doses of the same GnRH-agonist molecule, the Triptorelin, with the aim to identify any diversity in ovarian performance and in the number of good embryos.

3.1.2 MATERIALS AND METHODS

We enrolled in the egg donation program, 60 oocyte donors who undergone controlled ovarian hyperstimulation with GnRH antagonist at the IVI clinic of Barcelona. The recruitment period is between February and May 2015.

We identified three arms of randomization, with identification of three different treatment groups, each consisting of 20 clinical cases:

- a) Triptorelin 0.1 mg
- b) Triptorelin 0.2 mg
- c) Triptorelin 0.3 mg

The enrollment was carried out by following the protocols of IVI clinic, in accordance with the provisions of the Royal Decree, legislative document regulating egg donation in Spain. Each patient underwent a preliminary clinical investigation, with a focus on family history, in order to identify hereditary diseases (chromosomal disorders, genetic disorders, psychiatric disorders, etc ...) representatives factor of exclusion from the oocyte donation program.

Similarly, we investigated physiological, medical history, obstetrical and gynecological history of each patient, and were specified any previous eggs donation cycles.

During the first visit, the patients were informed of the objectives and requirements of the study and was delivered their informed consent, got signed before any invasive tests. Similarly it was then assigned an identification number.

In a later meeting the patients had a gynecological examination, with associated pelvic transvaginal ultrasound evaluation, performed in the early follicular phase, in order to exclude gynecological diseases (malformations, ovarian diseases, etc ...) and to carry out the antral follicle count (AFC), fundamental parameter as strongly predictive of ovarian reserve, so the response to controlled ovarian stimulation treatments.

Inclusion Criteria	Exclusion criteria
Age 18-35 years	Polycystic ovary syndrome
18<BMI<28	Comorbidity
AFC >10	
Negative medical History	

Each patient underwent controlled ovarian stimulation with GnRH antagonist protocol, performed by daily administration of r-FSH (follitropin alfa-and-follitropin beta) from the 2nd or 3rd day of the menstrual cycle, with doses ranging from 150- 225 IU / day sc. GnRH antagonist was administered according the flexible scheme (ganirelix or cetrorelix 0.25 mg/die).

The final trigger was made when at least 3 follicles > 18 were available, by the administration of Triptorelin, with different doses, depending on the randomization arm. The oocyte collection was carried out 36 h after induction.

The main outcomes were the proportion of retrieved and mature oocytes.

The secondary outcomes were the proportion of fertilized oocytes (FR), proportion of good embryos (transferred + vitrified) in relation to obtained embryos, and the incidence of ovarian hyperstimulation syndrome (OHSS).

For the statistical analysis SPSS 17, Chi-square and Kruskal-Wallis tests were used, $p < 0.05$ considered significant.

3.1.3 RESULTS

Of a total of 60 patients enrolled, data for 51 patients were analyzed, randomized into three arms:

- 18 patients (35.3%) performed the final trigger with Triptorelin 0.1 mg;
- 17 patients (33.3%) with Triptorelin 0.2 mg
- 16 patients (31.4%) with 0.3 mg Triptorelin.

9 donors were excluded from the analysis, 2 because we did not complete the donation and 7 because we performed conventional IVF rather than ICSI.

The number of patients per arm is considered equivalent ($\chi^2 = 0.12$; $p = 0.943$; chi-squared test).

Randomization Class	N cases	Percentage
Triptorelin 0,1 mg	18	35,3
Triptorelin 0,2 mg	17	33,3
Triptorelin 0,3 mg	16	31,4
Totale	51	100

The average age of treated patients was 25.2 years (median 25 years) with a minimum of 18 and a maximum of 35 years. The data were also stratified for the randomization classes. No statistically difference was not observed in the three study groups ($p = 0.865$).

		N	media	SD	mediana	Min	Max
Age (yrs)	0,1 mg	18	25,6	5,0	27,0	18	35
	0,2 mg	17	24,7	2,8	25,0	21	33
	0,3 mg	16	25,4	4,9	24,5	19	34
	Total	51	25,2	4,3	25,0	18	35

The mean BMI of the analyzed population is equal to 22.2 kg / m², with a minimum of 18.3 and a maximum of 26.5 kg / m².

		N	media	SD	mediana	Min	Max
BMI (kg/m²)	0,1 mg	18	22,2	2,2	22,2	18,7	26,5
	0,2 mg	17	22,1	2,4	21,9	18,3	26
	0,3 mg	16	22,4	2,1	22,2	19	26,2
	Total	51	22,2	2,2	22,2	18,3	26,5

P=0,920

The mean duration of ovarian stimulation was 8.9 days (median 9 days), in line with what is commonly found in clinical practice. The average time, in days, of the administration of a GnRH-antagonist was equal to 3.9 days, a 4-day median, a minimum of 2 and a maximum of 5 days, with no difference between classes of randomization.

About the type of u-FSH administered, no significant differences were observed in the study population, without modification of results after sample stratified by randomization classes (*p=0,837*)

The mean total number of ovarian follicles found in ultrasound (US) evaluation on the triggering day was 23.4 (median 23 follicles), while the mean number of oocytes retrieved was a 18,8 follicles, with a mean of 14.7 MII oocytes.

	0,1 mg (media)	0,2 mg (media)	0,3 mg (media)	Totale (media)
N° tot follicoli	22,4	22,8	25,2	23,4
N° foll\geq14 mm	15,2	15,4	15,8	15,5
N° foll\geq16 mm	10,2	9,8	10,0	10,0
N° foll\geq18 mm	4,5	5,2	4,6	4,7
N° ovociti recuperati	18,4	19,9	17,9	18,8
N° ovociti maturi MII	14,9	13,9	15,4	14,7

The yield of ovarian response was calculated as the number of mature oocytes (MII) recovered in relation to the number of ovarian follicles with a diameter ≥ 14 mm measured at US evaluation. The average yield in patients amounted to 96.6% (median 92.3%), with no statistical significance.

The average number of fertilized oocyte was 10.6 and were obtained an average number of good embryos of 6.4 (median: 7 embryos). The mean number of embryos transferred was 1.7 embryos (median: 2 embryos)

Note: In 14 donors part of the oocytes recovered were inseminated and part vitrified, thus we lack some data on the number of fertilized eggs and the number of embryos obtained.

Primary Outcomes

Primary objective of the study was to assess whether different doses of the same agonist affect ovarian performance. In particular, the our primary outcomes were:

- number of oocytes retrieved in relation to the number of ovarian follicles ≥ 14 mm in diameter, reported on the trigger day. (COCs / foll ≥ 14)
- number of mature oocytes (MII) in relation to the number of oocytes retrieved at pick-up

Both the proportion of retrieved oocytes (COCs) in relation to follicle > 14 mm on the triggering day (122.3% group 1, 131.5% group 2, 114.3% group 3) and the proportion of

mature oocytes in relation to COCs (81% group 1, 76.6% group 2, 86.8% group 3) were comparable in the groups.

COCs/ fol\geq 14 mm		N	Media	SD	Mediana	Mín.	Máx.
	0,1 mg	18	122,3	36,1	117,1	68,8	190,0
	0,2 mg	17	131,5	56,9	112,5	73,3	244,4
	0,3 mg	16	114,3	31,5	117,7	55,6	193,8
	Totale	51	122,9	42,7	115,4	55,6	244,4

MII/ COC_s (%)		N	Media	SD	Mediana	Mín.	Máx.
	0,1 mg	18	81,0	14,6	81,2	57,9	100,0
	0,2 mg	17	76,6	21,9	80,0	31,1	100,0
	0,3 mg	16	86,8	13,1	88,5	58,3	100,0
	Total	51	81,3	17,2	84,2	31,1	100,0

Secondary outcomes

- a) fertilization rate
- b) proportion of good embryos (transferred + vitrified) in relation to obtained embryos
- c) OHSS rate

For the secondary outcome, we analysed data from 37 donors and in 14 the oocytes were vitrified.

a) Fertilization rate (oocytes fertilized / MII)

We observed the fertilization rate of 79.9%, with a median of 83.3%, a minimum of 33.3% and a maximum of 100%, with no significant difference in relation to the dose administered Triptorelin.

Fertilization rate (%)		<i>N</i>	<i>Media</i>	<i>SD</i>	<i>Mediana</i>	<i>Mín.</i>	<i>Máx.</i>
	<i>0,1 mg</i>	10	80,2	17,1	83,8	45,5	100,0
	<i>0,2 mg</i>	14	76,5	19,3	82,0	33,3	100,0
	<i>0,3 mg</i>	13	83,2	12,5	83,3	62,5	100,0
	<i>Totale</i>	37	79,9	16,4	83,3	33,3	100,0

p=0,799

b) Proportion of good embryos (transferred + vitrified) in relation to obtained embryos

The proportion of good embryos (transferred + vitrified) in relation to obtained embryos was 61,6% (57.7% group 1, 64.6% group 2, 61.4% group 3), with no differences in the three groups.

		<i>N</i>	<i>Media</i>	<i>SD</i>	<i>Mediana</i>	<i>Mín.</i>	<i>Máy.</i>
<i>Ovocitos útiles/ fecundados</i>	<i>0,1 mg</i>	10	57,7	23,4	57,9	22,2	88,9
	<i>0,2 mg</i>	14	64,6	20,9	72,1	29,4	87,5
	<i>0,3 mg</i>	13	61,4	25,8	71,4	0,0	90,0
	<i>Total</i>	37	61,6	22,9	66,7	0,0	90,0

p=0.833

c) OHSS rate

There were no cases of moderate to severe OHSS. Only in 4 cases, the patients reported abdominal discomfort, in the absence of additional clinical data pathognomonic of OHSS. In particular, one case was observed in the group of patients treated with Triptorelin 0.1 mg and 3 cases in the group treated with 0.2 mg Triptorelin for final oocyte maturation.

The results confirm that the use of the agonists in the final trigger is a valid pharmacological strategy in the prevention of moderate/severe forms of OHSS.

3.1.4 DISCUSSION

The use of GnRH-agonist (GnRH-a) for the trigger, as an alternative to hCG, today represents a valid therapeutic strategy in patients undergoing controlled ovarian stimulation. Their use has been made possible by the introduction, in the late '90s, of the GnRH antagonists (GnRH-door) to prevent premature LH surge in IVF cycles.

Several clinical studies published in the literature have demonstrated their efficacy in the induction of final oocyte maturation, because able to induce an LH peak. In particular, the GnRHa induced LH-surge consists of two phases: a short ascending limb (> 4 h) and a long descending limb (> 20 h), for a total of 24–36 h (Itskovitz et al., 1991). In contrast, the mid-cycle surge of the natural cycle (Fig. 1) is characterized by three phases: a rapidly ascending phase lasting for 14 h, a plateau of 14 h and a descending phase of 20 h, in total 48 h (Hoff et al., 1983).

It was also observed that the GnRH-a trigger, simultaneously defines a peak of FSH, similar to what occurs in the natural cycle, responsible for the formation of LH receptors in luteinized granulosa cells, the oocyte nuclear maturation and cumulus expansion (Stickland e Beers, 1976; Eppig, 1979; Zelinski-Wooten et al., 1995;. Yding Andersen et al., 1999;. Yding Andersen, 2002), all aspects that probably correlate with the highest number of mature oocytes MII retrieved (Imoedemhe et al, 1991a; Humaidan et al, 2005, 2009a, 2010; Oktay et al, 2010).

Their use in clinical practice is mainly due to the need to reduce the incidence of ovarian hyperstimulation syndrome (OHSS), still one of the complications associated with ovarian stimulation in IVF cycles.

In a meta-analysis published in 2011, a significantly lower incidence of OHSS in patients treated with agonist vs hCG was observed (OR 0.10 , 95% CI 0.01 to 0.82, I² = 0%, P = 0.74; 5 studies). Youssef at al., in 2011 had similar results in a RCT conducted in patients undergoing ovarian stimulation for egg donation (OR 0.06, 95% CI 0.01to0.31, I² = 0%, P = 0.96; 3 trials).

Despite the potential advantages by the use of GnRh agonist to trigger final oocyte maturation, several studies reported a poor clinical outcome with an extremely high early pregnancy loss rate when GnRHa was used to trigger ovulation (Fauser et al., 2002; Humaidan et al., 2005; Kolibianakis et al., 2005). The poor results were attributed to a luteal phase insufficiency despite standard luteal phase support (LPS) with progesterone and estradiol. Thus, until now

the greatest advantage of GnRHa triggering in ovarian hyperstimulation, is the total elimination of OHSS. This has led to the fact that GnRHa triggering is now the method of choice in many oocyte donation programs, resulting in a high good quality oocyte yield, an elimination of OHSS, a higher degree of patient convenience and an excellent pregnancy rate in recipients (Hernandez et al., 2009; Bodri et al., 2009).

Several studies proposed different molecules and different doses of GnRha to trigger final oocyte maturation.

The aim of our study is to identify the optimal dose of the same GnRH agonist for the induction of final oocyte maturation. In our knowledge, this is the first randomized clinical trial that compare different doses of the same GnRh agonist to trigger final oocyte maturation. Our data show an average number of MII oocytes mature equal to 78% of the total of oocytes, with an ovarian efficiency (defined as the number of oocytes retrieved in relation to follicles ≥ 14 mm in the triggering day) of 96.6%.

The proportion of retrieved oocytes (COCs) in relation to follicle >14 mm on the triggering day (122.3% group 1, 131.5% group 2, 114.3% group 3) and the proportion of mature oocytes in relation to COCs (81% group 1, 76.6% group 2, 86.8% group 3) were comparable in the groups. The results confirm that the use of GnRH agonists 36 hours before the oocyte retrieval contributes largely to the maturing oocyte.

For the seconds endpoints, the FR (80.2% group 1, 76.5% group 2, 83.2% group 3) and the proportion of good embryos (transferred + vitrified) in relation to obtained embryos (57.7% group 1, 64.6% group 2, 61.4% group 3) didn't differ significantly between the groups.

Finally, no OHSS cases occurred in any of the groups.

Performing an analysis of the individual arms of the randomization, we observed that the arm associated with the minimum dose of Triterelin (0.1 mg) showed the same results of the groups treated with higher doses of the drug (0.2 mg and 0.3 mg). If this result was confirmed in subsequent surveys, it could represent primarily an advantage in terms of costs, with a reduction in health care costs.

At the same time, it might be proposed an extension of the protocol in homologues IVF cycles: in this way it might be interesting to see whether, a reduction of GnRh agonist doses could have less negative impact on the luteal phase.

Final trigger with 0,1 mg of Triptorelin could represent a standard dose, more safe not only in terms of OHSS rate reduction, but also for the luteal phase, improving the clinical and ongoing pregnancy rate, with a possible reduction of miscarriage rates.

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3.2. Long term COH complications: metabolic, cardiovascular and oncological risk evaluation. An observational, retrospective study

3.2.1 BACKGROUND

Over the years, the scientific community has always paid attention to the possible side effects of these drugs, and in particular the possibility of developing cancer, particularly ovarian, breast and endometrium. It is well known that hormonal factors may be involved in the etiology of certain cancers, in particular those of the female reproductive system.

A significant number of scientific papers published in the literature has tried to address the possible long-term effects of ovulation-inducing drugs on cancer risk. Although early cohort findings raised concern regarding effects on ovarian cancer (1, 2), more recent findings have been largely reassuring (3, 4–6). Results regarding breast cancer are hard to interpret given that in the largest studies the risks have ranged from inverse relationships (7) to increased risks (4, 6) to no associations (9–14). The one site for which there is some consistency is that of endometrial cancer, with a number of studies suggesting possible risk increases (4, 10, 11, 15, 16).

Brinton LA. et al, in a retrospective cohort study, concluded that there were no significant relationships of IVF exposures to the risks of breast, endometrial, or ovarian cancers. However, compared with women with no fertility treatment, the HR for ovarian cancer associated with IVF was 1.58 (95% confidence interval [CI] 0.75–3.29), with higher risk among those receiving four or more cycles (HR 1.78, 95% CI 0.76–4.13). There was also a nonsignificantly elevated risk for endometrial cancer among women who received 1–3 IVF cycles (HR 1.94, 95% CI 0.73–5.12), but additional cycles were associated with less risk. In contrast, the risk of in situ cervical cancer was significantly reduced and invasive cervical cancer nonsignificantly reduced among women receiving IVF as well as other fertility treatments.

van den Belt-Dusebout, in a recent study published in 2016, concluded that Breast cancer risk in IVF-treated women was not significantly different from that in the general population (SIR, 1.01 [95% CI, 0.93–1.09]) and from the risk in the non-IVF group (HR, 1.01 [95% CI, 0.86–1.19]). The cumulative incidences of breast cancer at age 55 were 3.0% for the IVF group and 2.9% for the non-IVF group ($P = .85$). The SIR did not increase with longer time since treatment (≥ 20 years) in the IVF group (0.92 [95% CI, 0.73–1.15]) or in the non-IVF group (1.03 [95% CI, 0.82–1.29]). Risk was significantly lower for those who underwent 7 or more

IVF cycles (HR, 0.55 [95% CI, 0.39-0.77]) vs 1 to 2 IVF cycles and after poor response to the first IVF cycle (HR, 0.77 [95% CI, 0.61-0.96] for <4 vs ≥ 4 collected oocytes).

About the cardiovascular and metabolic risk in patients undergoing IVF, to date there are few data in literature. It consists mostly of data collected only in cases of ovulatory infertility. In particular, in patients undergoing homologous IVF cycles, it was observed that PCOS patients have seven times increased risk of developing myocardial infarction (18). Moreover, the condition of oligomenorrhea, typical of the syndrome, it is seen to be associated with an increased risk of cardiovascular disease (CVD) (19).

Recent studies report a temporary increase of blood pressure associated to the use of oral contraceptives, as well as to a potentially higher risk of developing cardiovascular disease. (20-21). Potential mechanisms involved in the elevation of blood pressure probably involve the renin-angiotensin system: in particular, in patients taking CO was observed an elevation of angiotensinogen levels along with an abnormal activation of the renin systems.

About the cardiovascular and metabolic risk of patients undergoing IVF, recent studies report an increased risk of preeclampsia, which is also associated with an increased risk of preterm birth, gestational diabetes, postpartum hemorrhage and low birth weight, with risk levels ranging from + 20% to + 60% compared to pregnancies arisen spontaneously (22). In relation to the mode of delivery, caesarian-section increases of + 50% compared to spontaneous vaginal delivery.

It is well established that advanced maternal age is one of the risk factors that most correlates with adverse maternal and fetal outcomes. A recent meta-analysis showed that the risk of developing preeclampsia and gestational hypertension is greatly increased in pregnancies obtained from egg donation compared to those obtained by homologous IVF techniques (OR 2.54; 95% CI; $p < 0.001$) or by spontaneous conception (OR 4.34 ; 95% CI; $p < 0.001$) (23).

Similar data were also confirmed by other studies in the literature (24,25). In pregnancy obtained from egg donation it would seem to trigger an immune response against maternal fetal allogeneic antigens, responsible for abnormal placentation. This leads to the release into the maternal circulation of anti-angiogenic and pro-inflammatory mediators that are wing behind the development of endothelial dysfunction leading to increased peripheral vascular resistance.

No more data we found about long term risks in IVF patients.

3.2.2 AIMS OF THE STUDY

Retrospective observational study to assess a possible interlinkages between To assess long-term risk of cardiovascular, metabolic and oncological complications after ovarian stimulation for IVF. We tried to investigate too a possible correlation between the onset of pregnancy complication and cardiovascular and metabolic disease after a long time from IVF treatments

3.2.3 MATERIAL AND METHODS

We enrolled all patients who undergone 2 ore more homologues IVF treatments from 2000 to 2010 at the IVI clinic of Barcelona.

We created an "ad hoc questionnaire" containing epidemiological data (BMI, age, etc.) and current medical history. The information relating to the treatment phase were recovered from elettronic database of IVI clinic (Sivis).

In particular, we collected data related to:

- infertility duration
- type of infertility
- causal factor
- type of protocol used
- days of stimulation
- total dose of gonadotropins
- number of oocytes retrieval
- number of transferred embryos
- eventual diagnosis pre facility
- fertilization rate
- implantantion rate
- clinical pregnancy rate
- Cycle number successfully

3.2.4 PRIMARY ENDOPOINTS

- Hypertension disease
- Diabetes
- Dyslipidemia

SECONDARY ENDPOINTS:

- Breast cancer
- Gynaecological cancer (endometrial cancer, ovarian cancer)

Results

In order to carry out a scientific work with a good robustness from the statistical point of view, we decided to collect all the data related to homologous IVF cycles carried out from 2000 to 2016. Therefore, we are waiting the last born conceived in 2016 for the completion of the data analysis.

QUESTIONNAIRE

QUESTIONARIO ANAMNESTICO

NOME _____ COGNOME _____ DATA NASCITA _____

RESIDENZA _____

RECAPITO TELEFONICO _____

PROFESSIONE _____

ETNIA _____ PESO _____ ALTEZZA _____ BMI _____

TIPO ALIMENTAZIONE: ☐normale ☐vegetariana ☐vegana ☐altro

ANAMNESI FAMILIARE

Iperensione arteriosa		TVP	
Diabete I		Trombofilia congenita	
Diabete II		Carcinoma mammario	
Infarto miocardico		Carcinoma ovarico	
Ipercolesterolemia		Patologie malformative	
Ev. cerebrovascolari		Altro (specificare)	

P padre	Na.P nonna paterna
M madre	Na. M nonna materna
F fratello	Z.P zio paterno
S sorella	Za. P zia paterna
N.P nonno paterno	Z.M zio materno
N.M nonno materno	Za.P zia materna

ANAMNESI PATOLOGICA REMOTA

Iperensione arteriosa		TVP	
Diabete I		Trombofilia congenita	
Diabete II		Carcinoma mammario	
Infarto miocardico		Carcinoma ovarico	
Ipercolesterolemia		Patologie malformative	
Ev. cerebrovascolari		Altro (specificare)	

- INTERVENTI CHIRURGICI

- FARMACI ASSUNTI

ANAMNESI GINECOLOGICA-OSTETRICA

ETA' MENARCA: _____ DURATA CICLO _____ gg

RITMO CICLO:

- ☐ Eumenorrea (Ritmo min...../ Ritmo max.....)
- ☐ Oligomenorrea (Ritmo min / Ritmo max.....)
- ☐ Amenorrea (specificare da quando: _____)
- ☐ Polimenorrea (Ritmo min... / Ritmo max...)

QUANTITA' CICLO: ☐ Normale ☐ Abbondante ☐ Scarso

CONTRACCZIONE: ☐ SI ☐ NO Durata: _____

Tipo contraccettivo :

- ☐ Pillola EP
- ☐ Pillola solo progestinico
- ☐ Anello vaginale
- ☐ Spirale al rame
- ☐ Spirale medicata
- ☐ Impianto sottocutaneo
- ☐ Altro (specificare)

☐ Gravidanze spontanee n. _____

☐ Gravidanze da PMA n. _____

INFERTILITÀ

☐ PRIMARIA ☐ SECONDARIA ANNI sterilità _____

CAUSE DI STERILITA'

- ☐ Fattore Maschile
- ☐ Sdr ovaio policistico
- ☐ Fattore tubarico
- ☐ Anovulazione
- ☐ Disordini genetici
- ☐ Endometriosi se sì , interventi _____

TRATTAMENTI PMA

I LIVELLO -INSEMINAZIONI INTRAUTERINE -

- ☐ Omologa; n.° cicli _____
☐ Eterologa; n.° cicli _____

GRAVIDANZA NO

GRAVIDANZA SI* (specificare sotto)

Gravidanza avvenuta al ciclo n.° _____ età al concepimento _____ anno di attuazione _____
 Gravidanza avvenuta al ciclo n.° _____ età al concepimento _____ anno di attuazione _____
 Gravidanza avvenuta al ciclo n.° _____ età al concepimento _____ anno di attuazione _____

II LIVELLO - FECONDAZIONE IN VITRO-

- ☐ Omologa; n.° cicli _____
☐ Eterologa; n.° cicli _____ da ☐ ovodonazione ☐ donazione di seme ☐ doppia donazione

GRAVIDANZA NO

GRAVIDANZA SI* (specificare sotto)

Gravidanza avvenuta al ciclo n.° _____ età al concepimento _____ anno di attuazione _____
 Gravidanza avvenuta al ciclo n.° _____ età al concepimento _____ anno di attuazione _____
 Gravidanza avvenuta al ciclo n.° _____ età al concepimento _____ anno di attuazione _____

- ☐ ABORTI: n.° _____ settimane _____ anno _____
 n.° _____ settimane _____ anno _____
 n.° _____ settimane _____ anno _____

INTERRUZIONI VOLONTARIE DI GRAVIDANZA SI NO

Settimane gestazionali _____

Art. 4 Età _____ n.° _____

Art. 6 Età _____ n.° _____ causa _____

- ☐ GRAVIDANZE ECTOPICHE n.° _____ Anno _____
 n.° _____ Anno _____

TIPOLOGIA PARTO

☐ PARTO SPONTANEO EPOCA GESTAZIONALE _____

parto n.° _____ settimane gestazionali _____ anno _____

parto n.° _____ settimane gestazionali _____ anno _____

parto n.° _____ settimane gestazionali _____ anno _____

TAGLIO CESAREO:

cesareo n.° _____ settimane gestazionali _____ anno _____

cesareo n.° _____ settimane gestazionali _____ anno _____

cesareo n.° _____ settimane gestazionali _____ anno _____

Indicazione al taglio cesareo n.° _____ :

Indicazione al taglio cesareo n.° _____ :

Indicazione al taglio cesareo n.° _____ :

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